Structural Comparison of Crustacean, Potato, and Mushroom Polyphenol Oxidases†

Jon S. Chen,‡ James F. Preston,§ Cheng-i Wei,*,‡ Parvin Hooshar,‡ Richard A. Gleeson,∥ and Maurice R. Marshall*,‡

Food Science and Human Nutrition Department, Department of Microbiology and Cell Science, and Whitney Marine Laboratory, University of Florida, P.O. Box 110370, Gainesville, Florida 32611-0370

Polyphenol oxidases (PPOs) from crustaceans (lobster, white and brown shrimp), potato, and mushroom were compared with respect to isozyme forms, molecular weight (mass), secondary structure, and immunochemical properties. Studies using nondenaturing polyacrylamide gel electrophoresis showed varied multiple forms of isozyme with different molecular weights existed among these enzymes. When competitive enzyme-linked immunoabsorbent assay (ELISA) and immunoblotting techniques were employed, these PPOs were shown to share similar antigenic determinants. They, however, varied in their secondary structure as revealed by spectropolarimetric analysis.

INTRODUCTION

Polyphenol oxidase (PPO) (EC 1.14.18.1), also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, and catecholase, is widely distributed in nature (Schwimmer, 1981). Unfavorable enzymatic browning caused by PPO on the surface of many plant and seafood products has been of great concern to food processors and scientists. Although melanin (blackening spot) formation does not affect the nutrient content of food products, it is perceived as spoilage by consumers. Economic loss resulting from this enzymatic action has always been significant and presents a practical problem to the food industry. Enzymatic browning of fruits, vegetables, and crustaceans due to PPO activity has been extensively studied (Walker, 1964; Macrae and Duggleby, 1968; Flurkey and Jen, 1978; Sciancalepore and Longone, 1984; Simpson et al., 1988; Ferrer et al., 1989; Chen et al., 1991a).

Recently Rolle et al. (1990), using circular dichroism (CD) spectropolarimetry, demonstrated differences in the secondary structure between endogenously and trypsin activated forms of Florida spiny lobster PPO. Chen et al. (1991a) showed that PPOs from plant and crustacean sources differed in their catalytic activity in the oxidation of DL-β-3,4-dihydroxyphenylalanine (DL-Dopa) and sensitivity to inhibition by kojic acid. Since the conformational structures of these fungal, plant, and crustacean PPOs were not well documented, this study was undertaken to elucidate whether conformational differences exist among these PPOs using immunological techniques and circular dichroism spectropolarimetry.

MATERIALS AND METHODS

Mushroom tyrosinase with an activity of 2200 units/mg of solid was purchased from Sigma Chemical Co. (St. Louis, MO). Russet potato was purchased from a local supermarket. White shrimp (*Penaeus setiferus*) and brown shrimp (*Penaeus aztecus*) were obtained from a local seafood store. Fresh Florida spiny lobster (*Panulirus argus*) tails were obtained from the Whitney Marine Laboratory (Marineland, FL) and transported in ice to the Food Science and Human Nutrition Department, University of Florida at Gainesville. Lobster cuticle, shrimp cephalothorax (head), and potato peel were frozen in liquid nitrogen and ground into a fine powder using a Waring blender. The ground powder was stored at −20 °C until needed.

Extraction and Purification of PPO. PPO was extracted and purified according to the procedure of Chen et al. (1991b). One part of ground powder was added to three parts (w/v) of 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl and 0.2% Brij 35 (Fisher Scientific Co., Orlando, FL). The extract was stirred for 30 min at 4 °C and the suspension centrifuged at 8000g (4 °C) for 30 min. The supernatant was then dialyzed at 4 °C overnight against three changes of 4 L of 0.05 M sodium phosphate buffer (pH 6.5).

Crude PPO preparation was further purified using a nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) system. Equipment utilized included a gel tube chamber (Model 175, Bio-Rad Laboratories, Richmond, CA) and a power supply (Model EPS 500/400, Pharmacia LKB Biotechnology Inc., Piscataway, NJ). A 1-mL aliquot of crude enzyme extract (lobster, shrimp, or potato) was applied to each of eight gel tubes (1.4 cm i.d. × 12 cm length) containing 5% acrylamide/0.13% bis(acrylamide) gel and subjected to a constant current of 10 mA/tube in a buffer (pH 8.3) containing 5 mM Tris-HCl and 38 mM glycine (Sigma Chemical Co., 1984). PPO was visualized using a specific enzyme–substrate staining method (Chen et al., 1991b); 10 mM DL-β-3,4-dihydroxyphenylalanine (DL-Dopa) in 0.05 M sodium phosphate buffer (pH 6.5) was used as substrate. After the migration of the enzyme relative to the dye front (R_f) was determined using one of the eight gels, the remaining gels were sectioned at regions having the same R_f value. The sectioned gels containing PPO were homogenized in 0.05 M sodium phosphate buffer (pH 6.5) with a Dounce tissue grinder (Wheaton, Millville, NJ). Following filtration through a Whatman No. 4 filter paper, the filtrate was concentrated using an Amicon stirred cell (Model 8050, Amicon Co., Danvers, MA) fitted with a YM 10 filter (Amicon). For mushroom PPO, the enzyme (0.25 mg of solid/mL) in 0.05 M sodium phosphate buffer (pH 6.5) was further purified following the previously described procedures (Chen et al., 1991b).

Protein Quantitation and Enzyme Purity Determination. The protein contents of PPO preparation were quantitated using the Bio-Rad protein assay kit with bovine serum albumin (Sigma) as standard. Enzyme purity was examined using a mini gel system (Mini-Protean II dual slab cell; Bio-Rad Laboratories, 1985). Mushroom, potato, and crustacean PPOs (20 μg of protein/well) were loaded into the sample well of polyacrylamide gel [7.5% acrylamide/0.2% bis(acrylamide)], and electrophoresis was carried out at constant voltage (200 V) in a buffer (pH 8.3) containing 25 mM Tris-HCl and 0.19 M glycine for 35 min. The purity of PPO preparations was examined by staining the gels

* Authors to whom correspondence should be addressed.

† Florida Agricultural Experiment Station Journal Series No. R-02188.

‡ Food Science and Human Nutrition Department.
§ Department of Microbiology and Cell Science.
∥ Whitney Marine Laboratory.

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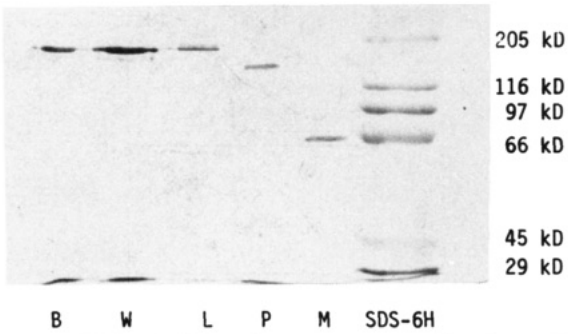

Figure 1. SDS–PAGE (without β-mercaptoethanol) profile of mushroom (M), potato (P), Florida spiny lobster (L), white shrimp (W), and brown shrimp (B) polyphenol oxidases (PPOs). SDS-6H containing carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa) was used as protein standard. Numerical designations represent molecular mass of the protein bands.

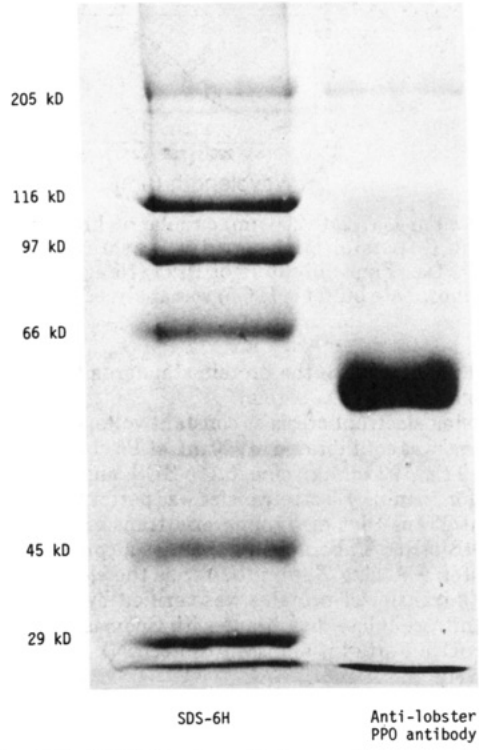

Figure 2. SDS–PAGE (under reducing condition) profile of anti-lobster PPO antibody. SDS-6H was used as protein standard.

with 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) and then with a Coomassie blue R-250 (Eastman Kodak Co., Rochester, NY) solution.

Enzyme Activity Assay. PPO activities were measured by adding 60 μL of enzyme preparations to 840 μL of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) and monitoring at 25 °C for 5 min. Maximal initial rate was determined as ΔA_{475} nm/min, and one unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25 °C. Unless otherwise specified, experiments were carried out three times in duplicate. The enzyme activities of mushroom, potato, lobster, white shrimp, and brown shrimp PPOs were determined as 96 000, 120 000, 4500, 1000, and 1200 units/mg of protein, respectively.

Antibody (IgY) Production and Purification. One milliliter of purified lobster PPO containing 100 μg of protein was used as an antigen for biweekly injection into a hen. Eggs laid by the immunized hen were collected, and antibody (IgY) was isolated and purified from the egg yolk (Polson et al., 1985). One part egg yolk was added to four parts (v/v) 0.1 M sodium phosphate buffer (pH 7.6). The mixture was made up to 3.5% (w/v) poly(ethylene glycol) (PEG) and stirred for 5 min. Following

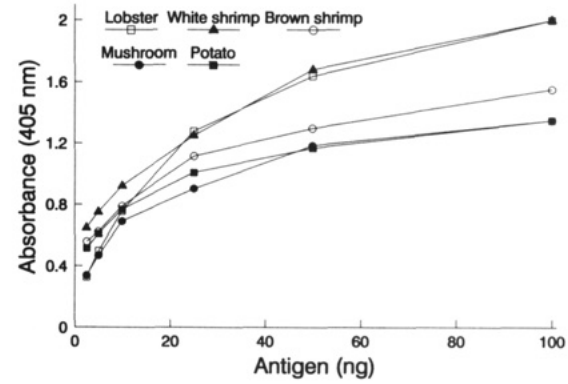

Figure 3. Titer determination of anti-lobster PPO antibody vs mushroom (●), potato (■), lobster (□), white shrimp (▲), and brown shrimp (○) PPOs. Mushroom, potato, lobster, white shrimp, and brown shrimp PPOs at 2.5–100 ng/well were used to react with anti-lobster PPO antibody at 2.5 μg/well.

centrifugation at 5000g (10 °C) for 20 min, the supernatant collected was made up to 8.5% PEG (w/v). The suspension was allowed to stand for 10 min followed by centrifugation at 5000g (10 °C) for 25 min. The pellet was dissolved in 2.5 volumes (v/v) of 0.1 M sodium phosphate buffer (pH 7.6), and the suspension was made up to 12% PEG (v/v). Again, the suspension was allowed to stand for 10 min and then centrifuged at 5000g (10 °C) for 25 min. The pellet was resuspended in one-fourth volume of 0.1 M sodium phosphate buffer (pH 7.6) and cooled to 0 °C before an equivalent volume of 50% ethanol (−20 °C) was added. Following centrifugation at 10000g (4 °C) for 25 min, the precipitate was dissolved in one-fourth volume of 0.1 M sodium phosphate buffer (pH 7.6) and the suspension dialyzed at 4 °C overnight against 4 L 0.1 M sodium phosphate buffer (pH 7.6). The dialyzed antibody preparation was made up to 0.1% NaN_3 and stored refrigerated until needed.

IgY Molecular Weight (Mass) Determination. Protein content of antibody preparation was also quantitated using the Bio-Rad protein assay kit. The molecular weight of antibody preparation was determined using sodium dodecyl sulfate–PAGE (SDS–PAGE, reducing condition) according to the method of Laemmli (1970). Mini slab gels (7 cm × 8 cm) at 1.0-mm thickness, consisting of stacking gel [4% acrylamide/0.1% bis(acrylamide)] and separating gel [7.5% acrylamide/0.2% bis(acrylamide)], were prepared for this study. Antibody preparations were diluted with 4 volumes of SDS sample buffer containing β-mercaptoethanol and heated for 4 min at 95 °C. After 30-μg aliquots were applied to each sample well, electrophoresis was carried out for 35 min at a constant voltage of 200 V. An SDS-6H high molecular mass protein standard (Sigma) containing carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa) was used. The methods of Weber and Osborn (1969) and Weber et al. (1972) were followed to estimate protein molecular weights.

IgY Titer Determination by Enzyme-Linked Immunosorbent Assay (ELISA). One hundred microliters of lobster PPO preparation containing 2.5–100 ng of protein in 0.1 $NaHCO_3$ (coating buffer, pH 8.6) was applied to sample wells of a microplate (Immulon 2, Dynatech). Following overnight incubation at 4 °C, the wells were aspirated and washed four times with PBS–Tween [0.01 M sodium phosphate (pH 7.4) containing 0.15 M NaCl and 0.2% (v/v) Tween 20] using the Nunc-Immuno Wash (A/S Nunc, Roskilde, Denmark). After 100 μL of primary antibody (0.01–10 μg) in PBS–Tween was added to each well, the plate was incubated at ambient temperature for 1 h. Aspirations and washings were repeated four times as previously described before 0.1 mL of secondary antibody (anti-chicken IgG–alkaline phosphatase conjugate, Sigma) was added. Following another 1-h incubation, the microplate was aspirated and washed again with PBS–Tween. Following the addition of 0.1 mL of *p*-nitrophenyl phosphate disodium (1.0 mg/mL) in assay buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, and 0.5 mM $MgCl_2$) to each well, the plate was further incubated at ambient temperature until color development. Absorbance at 405 nm was monitored using

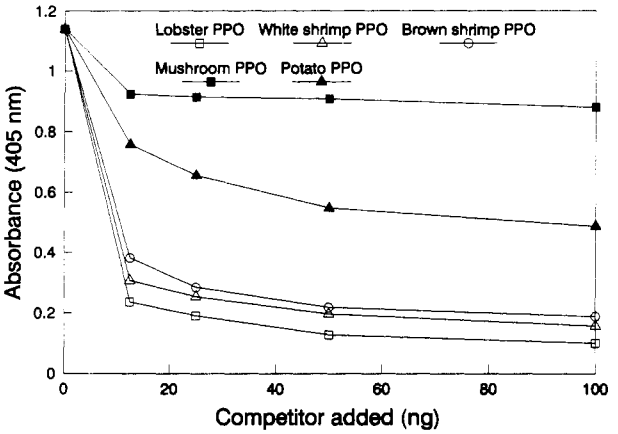

Figure 4. Analysis of antigenic properties of mushroom (■), potato (▲), lobster (□), white shrimp (△), and brown shrimp (○) PPOs by competitive ELISA. Lobster PPO (10 ng/well) as the antigen was plated and then reacted with anti-lobster PPO antibody–competitor mixtures containing 5 μg of anti-lobster PPO antibody and 12.5–100 ng of mushroom, potato, lobster, white shrimp, and brown shrimp PPOs.

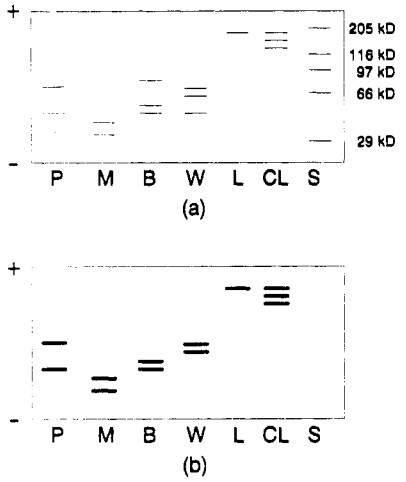

Figure 5. Protein profiles of electrotransferred standard (SDS-6H) and purified mushroom (M), potato (P), lobster (L), white shrimp (W), brown shrimp (B), and crude lobster PPO (CL) preparations under reducing condition on nitrocellulose membrane (a) and following staining with anti-lobster PPO antibody (b).

an ELISA reader (Model 2550, Bio-Rad). In this study, coating buffer without antigen was used as the control.

Analysis of Antigenic Properties of PPO. The competitive ELISA adopted from Seymour et al. (1991) was employed to study whether PPOs from mushroom, potato, white shrimp, and brown shrimp possessed similar antigenic determinants as Florida spiny lobster. After the microplate had been coated with lobster PPO (10 ng/well) for 1 h at ambient temperature, a 100-μL aliquot of antibody–competitive PPO mixture was added. The antibody–PPO mixture was prepared by mixing the competitive PPO (lobster, white and brown shrimp, potato, and mushroom; 0.2–2.0 μg/mL) with an equal volume of primary antibody solution (10 or 20 μg/mL of IgY) for 1 h at ambient temperature. The assay procedures were conducted as previously described for the ELISA.

Immunoblotting. Purified mushroom, potato, lobster, white shrimp, and brown shrimp PPOs and crude lobster PPO preparations were subjected to SDS–PAGE under reducing condition and then electrotransferred to nitrocellulose membrane (5 cm × 8 cm; Thomas Scientific, Swedesboro, NJ). Mini slab gels (7 cm × 8 cm) at 1.0-mm thickness, consisting of stacking gel [4% acrylamide/0.1% bis(acrylamide)] and separating gel [7.5% acrylamide/0.2% bis(acrylamide)], were prepared according to the Mini-Protean II dual slab cell instruction manual. One-hundred-nanogram aliquots of test samples were applied to each well and run with the protein standards (SDS-6H molecular weight marker kit, Sigma).

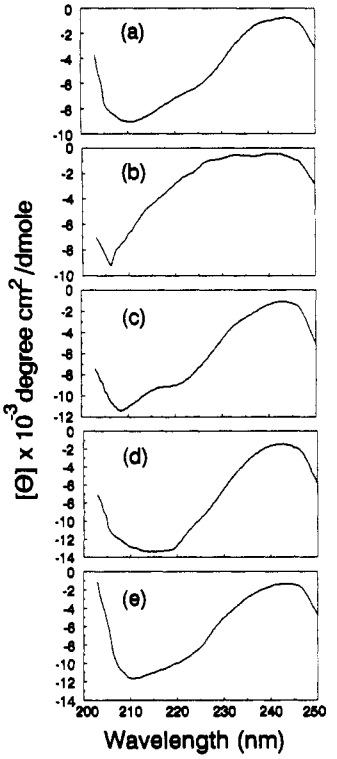

Figure 6. Far-UV (250–200 nm) circular dichroic spectra of (a) mushroom, (b) potato, (c) lobster, (d) white shrimp, and (e) brown shrimp PPOs. Four milliliters of PPO (15 μg/mL) in 0.5 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature.

Following electrophoresis at constant voltage of 200 V for 35 min, the gel was equilibrated in 200 mL of Towbin transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol; pH 8.3) for 15 min. Electrotransfer was performed according to the Mini Trans-Blot electrophoretic transfer cell instruction manual (Bio-Rad Laboratories, 1989) at a constant current of 100 mA for 1 h using Towbin buffer as the electrolytic buffer. Complete transfer of proteins was verified by staining the gel and the nitrocellulose membrane with Coomassie blue solution and AuroDye Forte kit (Janssen Biotech NV, Olen, Belgium), respectively.

Nitrocellulose membrane, following electrotransferring, was rinsed with phosphate-buffered saline (PBS) three times and then incubated with Blotto–Tween blocking solution (5% w/v nonfat dry milk, 0.2% v/v Tween 20, and 0.02% w/v NaN_3 in PBS; Harlow and Lane, 1988) at ambient temperature with agitation for 2 h. After two washes of 5 min each in PBS, the membrane was incubated for 1.5 h in the primary antibody (IgY) solution (10 μg/mL). Following washing with four changes of PBS for 5 min each, the membrane was treated for 1 h with the secondary antibody (anti-chicken IgG–alkaline phosphatase conjugate, Sigma) at the dilution of 1/2000. The membrane was then washed again with PBS as previously described and incubated with 100 mL of alkaline phosphatase buffer [100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris (pH 9.5)] containing 0.016% bromochloroindolyl phosphate (BCIP) and 0.032% nitro blue tetrazolium (NBT) (Harlow and Lane, 1988) for 30 min. The reaction was stopped by rinsing the membrane with PBS containing 20 mM EDTA.

Spectropolarimetric Analysis of PPO. The circular dichroic (CD) spectra of PPO were scanned at the far-UV (250–200 nm) range using a Jasco J-20 automatic recording spectropolarimeter (Japan Spectroscopic Co., Tokyo). A 1.0-cm Suprasil (Helma Cells) cuvette with a 1.0-cm light path was used. Four milliliters of PPO (15 μg/mL) in 0.5 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature. Calculations of the secondary structures were carried out by computer analysis of the spectra using the SSE program (Japan Spectroscopic Co.,

Table I. Secondary Structure Estimates of Various Polyphenol Oxidases (PPOs) from Far-UV Circular Dichroic Spectra[a]

PPO	% of secondary structure				
	α-helix		β-sheet	β-turn	random coil
mushroom	18.3 ± 0.2[b]	18.1 ± 0.3[c]	17.3 ± 0.3	24.8 ± 0.2	39.5 ± 0.3
potato	14.8 ± 0.1[b]	13.1 ± 0.2[c]	34.6 ± 0.2	28.4 ± 0.4	22.2 ± 0.2
lobster	24.4 ± 0.3[b]	25.1 ± 0.4[c]	26.2 ± 0.3	21.4 ± 0.1	29.9 ± 0.3
white shrimp	29.0 ● 0.4[b]	29.7 ± 0.3[c]	30.0 ± 0.1	11.3 ± 0.2	29.7 ± 0.3
brown shrimp	20.1 ± 0.2[b]	20.0 ± 0.1[c]	22.3 ± 0.3	15.2 ± 0.2	42.4 ± 0.1

[a] The circular dichroic spectra of PPO were scanned at the far-UV (250–200 nm) range. Four milliliters of PPO (15 μg/mL) in 0.5 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature. [b] Mean value ± standard deviation. [c] Calculated according to the formula proposed by Greenfield and Fasman (1969).

1985) with myoglobin, cytochrome *c*, ribonuclease A, lysozyme, and papain as CD references.

RESULTS AND DISCUSSION

SDS-PAGE Profile of PPO. Multiple forms of isozyme differing in molecular weights were observed for mushroom (five isozymes), potato (four isozymes), lobster (three isozymes), white shrimp (two isozymes), and brown shrimp (two isozymes) PPO preparations after the nondenaturing preparative polyacrylamide gel was stained with DL-Dopa (data not shown). After being subjected to SDS-PAGE (without treatment of β-mercaptoethanol), the isozymes of mushroom and potato PPOs chosen for this study were shown to have lower molecular masses than crustacean (lobster, white shrimp, and brown shrimp) PPOs, estimated as 66 and 148 kDa, respectively (Figure 1). These are in agreement with the previously reported data of Anisimov et al. (1978) and Bouchilloux et al. (1963). As to lobster, white shrimp, and brown shrimp PPOs, their molecular masses were determined as 200, 190, and 190 kDa, respectively. Brown shrimp PPO of 190 kDa was close to that of the Gulf brown shrimp PPO (210 kDa) (Madero and Finne, 1982). However, the molecular mass of white shrimp PPO determined in this study varied from that reported previously by Simpson et al. (1988). The use of different preparation and analytical methods between these studies could have contributed to such a discrepancy.

Antibody Production and Molecular Weight Determination. The production of lobster PPO-specific antibody did not occur until after the second boosting; peak activity occurred on day 18 (data not shown). Antibody activity decreased gradually with time following the third and fourth boostings. The serum eventually lost antibody activity after day 35. The animal apparently had become tolerant to the antigen after the third boosting.

Antibody produced on days 17 and 18 had the highest activity and was pooled for molecular mass determination. Three distinct bands were observed on the SDS-PAGE gel (Figure 2). The molecular mass for the lower band was estimated as 59 kDa, which was close to the value reported for the heavy chain of IgY (Jensenius et al., 1981).

Antibody (IgY) Titer Determination. The dose-related interactions of various PPOs (mushroom, potato, lobster, white shrimp, and brown shrimp) at 2.5–100 ng with lobster PPO-specific antibody at 2.5 μg are shown in Figure 3. All PPOs studied showed affinity for the antibody. Therefore, these PPOs were partially cross-reactive and shared similar antigenic determinants. The appropriate dose range of the interaction of the various PPOs with the antibody was determined to be 2.5–10 ng/well. When the PPO was added at more than 10 ng/well, the antigen–antibody reaction was saturated and the sensitivity of the ELISA did not increase any further.

Immunological Characteristics of PPO. The absorbance of the lobster PPO–antibody reaction mixture in the presence of various competitors (lobster, white shrimp, brown shrimp, mushroom, and potato PPOs) at 405 nm as determined by ELISA is illustrated in Figure 4. The color intensity was reduced by 18–79% of the control (Abs = 0.967) when the various competitor–antibody mixtures containing 12.5 ng of competitor and 5 μg of antibody per sample well were added to the microplate. The increase of the competitor up to 100 ng/well slightly reduced the color intensity of the assay system. For mushroom and potato PPOs, their competition with lobster PPO for antibody was not enhanced even when both were added at 100 ng/well. Similar results were obtained when antibody content was increased to 10 μg/well (data not shown). The results again indicated that these PPOs were partially cross-reactive and therefore shared some similar structural components. However, definite structural differences are also noted because of the lack of competition with these two PPOs compared to PPO from crustaceans. From the extent of competition for lobster PPO-specific antibody, white shrimp and brown shrimp PPOs were shown to share more antigenic similarities to lobster PPO than potato and mushroom PPOs (in descending order).

Complete transfer of PPO bands along with protein standards from acrylamide gel onto nitrocellulose membrane was achieved following electrotransferring; the staining of the treated gels with Coomassie blue revealed no protein bands retained on the gel (data not shown). The purified white and brown shrimp, potato, and crude lobster PPO preparation had three protein bands, while the purified lobster PPO had one protein band (Figure 5a). Two protein bands were seen with the purified mushroom PPO (Figure 5a). The nitrocellulose membrane, following staining with lobster PPO-specific antibody, was found to contain dark bands corresponding to purified lobster, white shrimp, brown shrimp, mushroom, and potato PPOs and crude lobster PPO preparation. The white shrimp, brown shrimp, potato, mushroom, and potato PPOs had two dark bands, and the crude lobster PPO preparation had three (Figure 5b). Only one dark band was observed for the purified lobster PPO, and no dark band was found with the protein standards (Figure 5b). The results thus verified our previous findings that PPOs from potato, mushroom, and crustaceans shared similar structural components.

Spectropolarimetric Analysis of PPO. Lobster, white shrimp, and brown shrimp PPOs had similar circular dichroic spectra patterns (Figure 6c–e) but were different from those of mushroom and potato PPOs (Figure 6a,b). They all varied in their secondary structure (α-helix, β-sheet, β-turn, and random coil) (Table I). For example, the white shrimp PPO had a higher percentage of α-helix than brown shrimp PPO; they both showed the same broad negative ellipticity between 207 and 220 nm. The percentage of α-helix for mushroom, potato, and crustacean PPOs as determined by the SSE program was further verified by the values obtained when the formula of Greenfield and Fasman (1969) (Table I) was used. Crustacean

PPOs, in general, had a higher percentage of α-helix and lower percentage of β-turn than mushroom and potato PPOs (Table I). Although the percentages of the secondary structure of these PPOs estimated from the SSE program may not represent the absolute values, results from this study indicated that PPOs from various sources possessed varied secondary structure. With respect to structural similarities, the crustacean-producing PPOs were more closely related than mushroom and potato PPOs.

Conclusion. Differences in the secondary structure (α-helix, β-sheet, β-turn, and random coil) among polyphenol oxidases from mushroom, potato, Florida spiny lobster, white shrimp, and brown shrimp were verified by spectropolarimetric analysis. Using immunological assays, these PPOs were shown to share similar antigenic determinants when they were tested against antibody (IgY) prepared from hen immunized with lobster PPO.

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